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LONG TERM STORAGE OF ANTIBODY SENSITIZED MILLITITER™ IMMUNOASSAY PLATES FOR THE IDENTIFICATION AND QUANTITATION OF *FRANCISELLA TULARENSIS*

by

Yunus M. Siddiqui and Roberta E. Fulton

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August 1989

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ABSTRACT

In this report we have described a modified procedure for the "sandwich" fluorogenic enzyme-linked immunosorbent assay (FELISA) for the detection and quantitation of antigen, in which immunoassay plates were pre-coated with capture antibody or, alternatively, with capture antibody and blocking reagent, and stored frozen for periods up to 1 yr. Using *Francisella tularensis* as a model, two different identification protocols were employed. In the first, antibody-sensitized immunoassay plates stored frozen at -70°C were challenged with whole cells or with outer membrane protein of *F. tularensis*. In these experiments, there was no loss of sensitivity for the detection and quantitation of the antigen, when compared with freshly prepared immunoassay plates. This procedure took 5 h compared to more than 6 h required for performance of the assay using freshly prepared plates. In the second protocol, antibody-coated immunoassay plates were blocked with bovine serum albumin and stored frozen at -70°C for periods of up to 1 yr. This latter procedure shortened the assay time to 3 h while maintaining the same degree of sensitivity (10 fg (10^{-14} g) mL^{-1} of outer membrane protein and 100 colony forming units mL^{-1} of *F. tularensis* whole cells) as that achieved with freshly coated plates.

KEY WORDS: Nitrocellulose membranes, Enzyme-linked immunosorbent assay, Fluorogenic, *Francisella tularensis*, Outer membrane protein.

INTRODUCTION

Immunoassay methods for the detection/identification of potential biological warfare (BW) agent must be simple, reliable and rapid. However, the fluorogenic enzyme-linked immunosorbent assay (FELISA), as currently utilized for the identification of microorganisms, either antigenic fractions or whole cells, requires long incubation periods of 1 to 3 h to coat the solid phase with antibodies and to block non-specific reaction sites (1,2). These problems have been overcome in some commercially available identification kits by pre-blocking the non-specific sites, but most of these assay systems do not provide a quantitative measurement of antigens.

In this report, we have described a modification of the FELISA in which the length of time required for performance of the assay has been reduced. The protocol utilized antibody-sensitized immunoassay plates stored at -70°C for periods up to 1 yr and a blocking step which was shorter than that previously reported (1,2). The assay did not compromise sensitivity and required 5 h to complete. In addition, antibody-coated plates blocked with bovine serum albumin (BSA) and stored for periods of up to 1 yr, showed similar sensitivity and results were obtained in approximately 3 h.

MATERIALS AND METHODS

Reagents

Francisella tularensis rabbit antiserum was purchased from Sylvana (GIBCO/BRL Canada Ltd., Burlington, Ontario). Alkaline phosphatase-labelled goat anti-mouse IgG, BSA (fraction V), diethanolamine (DEA), and 4-methylumbelliteryl phosphate (4-MUP) were purchased from Sigma Chemical Company (St. Louis, Mo.). Tween-20 was purchased

from Bio-Rad Laboratories (Richmond, Ca.). Phosphate buffered saline (PBS) tablets (pH 7.4) were purchased from Oxoid Canada Ltd., (Ottawa, Ontario).

Preparation of Antigens

Outer membrane protein (OMP) and formalinized whole cells of *F. tularensis* (live vaccine strain) (LVS) were prepared as described elsewhere (2).

Preparation of Reagents

Immunoassay reagents were prepared and diluted as reported previously (2), with the exception that the blocking buffer consisted of PBS containing 3% BSA and 0.1% Tween-20. Anti-*F. tularensis* antibodies made in rabbit were purified by the method of Volk et al. (3). Mouse hyperimmune ascites fluids against OMP of *F. tularensis* were prepared and purified as described previously (2).

Immunoassay Plates

Millititer™-HA (nitrocellulose membrane) immunoassay plates and the Millititer™ filtration system were purchased from Millipore Corporation (Bedford, Mass.).

Immunoassay Procedure

The "sandwich" FELISA was carried out as previously described (2) with the exception that, after overnight coating of capture antibody on the nitrocellulose membrane solid phase and subsequent washing to remove excess antibody, the bottom surfaces of the wells

were blotted dry and some plates were sealed with Saran Wrap™ and stored at -70°C. The remaining immunoassay plates were incubated with blocking buffer (PBS + 3% BSA + 0.1% Tween-20) for 2 h prior to washing, subsequent blotting of bottom surfaces, wrapping and storage at -70°C.

At suitable time intervals thereafter, plates were removed from storage, incubated at 37°C for 15 min, and the remaining steps of the assay completed. Unblocked plates were blocked for 2 h, as described above, prior to completion of the assay. Controls consisted of plates which were freshly coated with capture antibody and subsequently blocked, with no intervening storage step prior to completion of the assay.

Relative fluorescence was measured directly on Millititer™-HA plates by a MicroFluor™ fluorometer (Dynatech Laboratories, Alexandria, Va.). Results were considered positive if the mean fluorescence reading minus one standard deviation (sd) was equal to or greater than the mean fluorescence reading of the negative control plus 2 sd.

RESULTS

Antibody-coated immunoassay plates coated with antibody and blocked prior to storage at -70°C, were assayed for immunoreactivity of the antibody at 1,3,6,9 and 12 months. The system was challenged with log dilutions of *F. tularensis* OMP (Figure 1, a-e) or whole cells (Figure 2, a-d) diluted in blocking buffer. Regardless of the length of storage, the detection limit for *F. tularensis* OMP (Table I) and for whole cells (Table II) in plates which were pre-coated with antibody, and in those which were pre-coated with antibody and blocked, prior to storage at -70°C, was the same as that achieved

in control plates which were freshly prepared. The detection limit for *F. tularensis* OMP was consistently observed to be 10^{-14}g mL^{-1} (500 ag per test volume). The lower limits of detectability for formalinized whole cells was 100 colony forming units (CFU) mL^{-1} (5 CFU per test volume).

DISCUSSION

A procedure that reduces the time required to perform the "sandwich" FELISA for the detection and quantitation of *F. tularensis*, without compromising sensitivity, has been described. Pre-coating immunoassay plates with capture antibody, or with capture antibody and blocking reagent, and storing them frozen, even for a period of 1 yr, did not diminish the immunoreactivity of the antibody. Using pre-coated plates, either blocked or unblocked, no significant difference in detection limit was observed when compared with freshly prepared immunoassay plates.

Attempts by others to demonstrate retention of assay sensitivity following short or long term storage of antibody-coated polystyrene immunoassay plates have proved unsuccessful. It has been reported that either the antibody is removed during washing or it becomes non-immunoreactive as a result of storage (4). Coulter et al. (5) reported a two-fold loss in sensitivity when antibody-coated plates were stored for 16 wk at 4°C. Considering the fact that polystyrene exhibits poor protein-binding characteristics (6) and that loss of immunoreactivity occurs following storage of antibody-sensitized plates, polystyrene would appear not to be an optimal choice as solid phase support for immunoassays. Nitrocellulose membrane, on the other hand, is well-known for its excellent protein-binding capacity (7,8,9,10). This characteristic combined with the fact, as demonstrated in this study, that antibody immunoreactivity and assay sensitivity are retained

following long term storage of antibody-coated Millititer™ plates (blocked or unblocked), would suggest that nitrocellulose is a good choice as solid phase support. In addition, since Millititer™ plates can be pre-coated with antibody (or antibody and blocking reagents) and stored for long periods without subsequent loss in assay sensitivity, large batches of plates could be prepared and stored for later use, thus reducing the total time required for performance of the assay.

We have described a modification of the FELISA procedure for the rapid detection and quantitation of *F. tularensis* (2). This modified procedure utilized immunoassay plates pre-sensitized with capture antibody, or capture antibody and blocking reagents, stored frozen for periods up to 1 yr. The assay did not compromise sensitivity and, once plates had been prepared and stored, the procedure required only 3 h to complete.

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TABLE I

Fluorescence counts (fl.ct.) and corresponding limits of detection of *F. tularensis* OMP by FELISA on antibody-sensitized plates stored at −70° C for periods up to 1 year. I. Freshly prepared (control) plates; II. antibody-coated plates; III. antibody-coated plates blocked with blocking reagent. Fluorescence counts are the mean and sd of six determinations on a single plate.

TEST INTERVAL (months)	I			II			III		
	NEGATIVE CONTROL + 2 sd (fl. ct.)	DETECTION LIMIT		NEGATIVE CONTROL + 2 sd (fl. ct.)	DETECTION LIMIT		NEGATIVE CONTROL + 2 sd (fl. ct.)	DETECTION LIMIT	
		TEST ± 1 sd (fl. ct.)	g mL^{-1}		TEST ± 1 sd (fl. ct.)	g mL^{-1}		TEST ± 1 sd (fl. ct.)	g mL^{-1}
1	138	164 ± 21	10^{-14}	145	169 ± 20	10^{-14}	135	147 ± 13	10^{-14}
3	128	147 ± 13	10^{-14}	107	139 ± 28	10^{-14}	158	198 ± 27	10^{-14}
6	137	157 ± 17	10^{-14}	123	139 ± 15	10^{-14}	158	183 ± 23	10^{-14}
9	152	193 ± 32	10^{-14}	181	238 ± 21	10^{-14}	199	223 ± 13	10^{-14}
12	163	198 ± 19	10^{-14}	196	222 ± 21	10^{-14}	214	252 ± 21	10^{-14}

TABLE II

Fluorescence counts (fl.ct.) and corresponding limits of detection of *F. tularensis* whole cells by FELISA on antibody-sensitized plates stored at −70° C for periods up to 1 year. I. Freshly prepared (control) plates; II. antibody-coated plates; III. antibody-coated plates blocked with blocking reagent. Fluorescence counts are the mean and sd of six determinations on a single plate.

TEST INTERVAL (months)	I			II			III		
	NEGATIVE CONTROL + 2 sd (fl. ct.)	DETECTION LIMIT		NEGATIVE CONTROL + 2 sd (fl. ct.)	DETECTION LIMIT		NEGATIVE CONTROL + 2 sd (fl. ct.)	DETECTION LIMIT	
		TEST ± 1 sd (fl. ct.)	CFU mL^{-1}		TEST ± 1 sd (fl. ct.)	CFU mL^{-1}		TEST ± 1 sd (fl. ct.)	CFU mL^{-1}
1	169	198 ± 28	100	206	241 ± 22	100	213	238 ± 24	100
6	517	590 ± 42	100	496	577 ± 31	100	495	540 ± 31	100
9	307	478 ± 63	100	372	575 ± 87	100	489	532 ± 30	100
12	630	697 ± 32	100	501	599 ± 91	100	506	570 ± 53	100

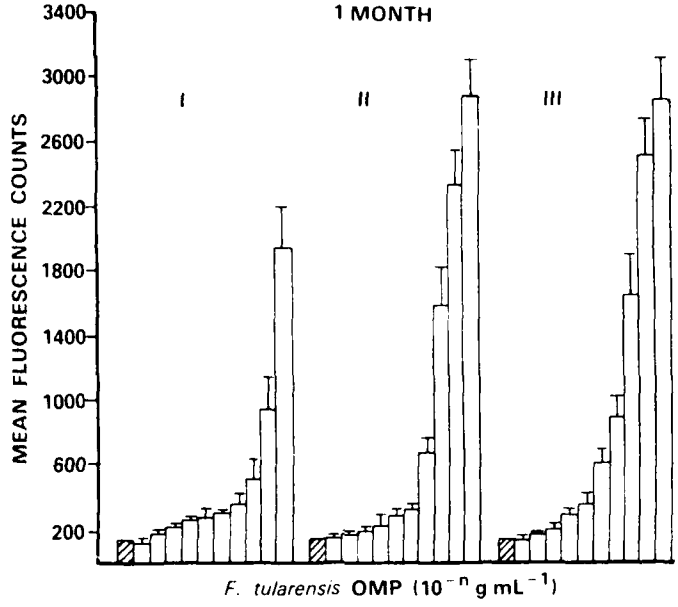


Figure 1a

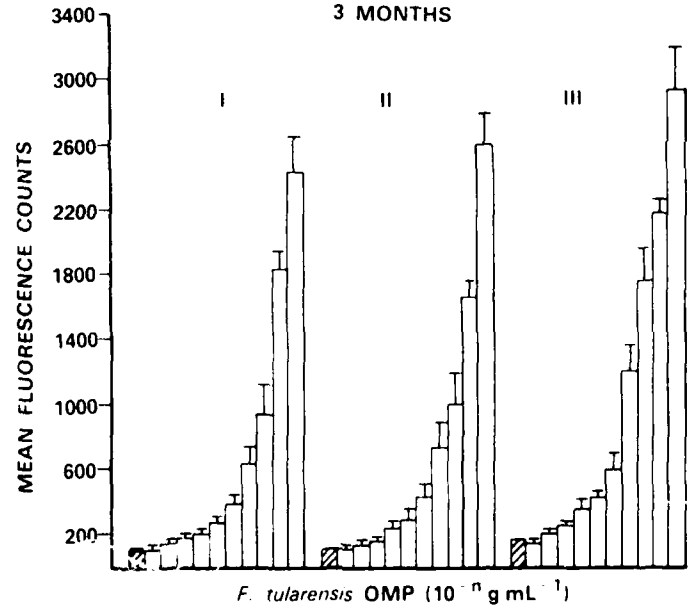
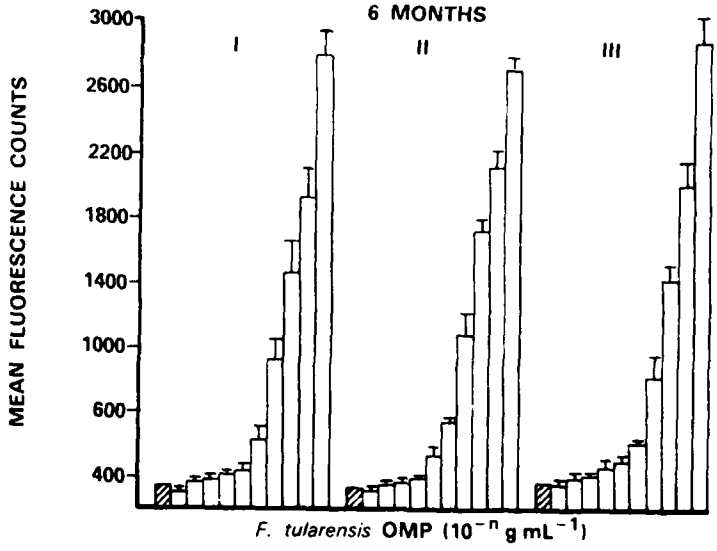


Figure 1b

Figure 1

Sensitivity of FELISA for the detection of *F. tularensis* OMP on antibody-sensitized plates stored at −70° C for periods up to 1 yr. The system was challenged with log dilutions of OMP (10^{-6} to 10^{-15} g mL^{-1}) (1 μg to 1 fg mL^{-1}) in blocking buffer. Freshly prepared (control) plates (I), antibody-coated plates (II) and antibody plates blocked with blocking reagent (III) were tested after 1, 3, 6, 9 and 12 months (a-e, respectively). Data points are the mean of six determinations on a single plate. Error bars represent sd of the mean. (▨) negative control plus 2 sd.



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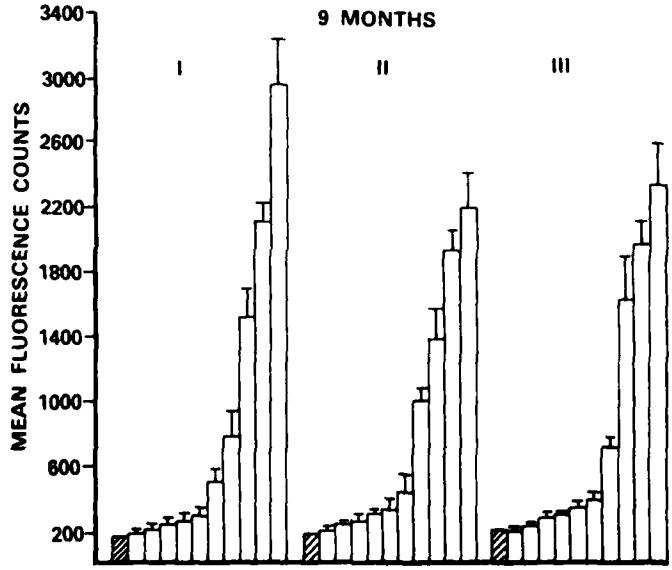

Figure 1d

Figure 1 (cont'd)

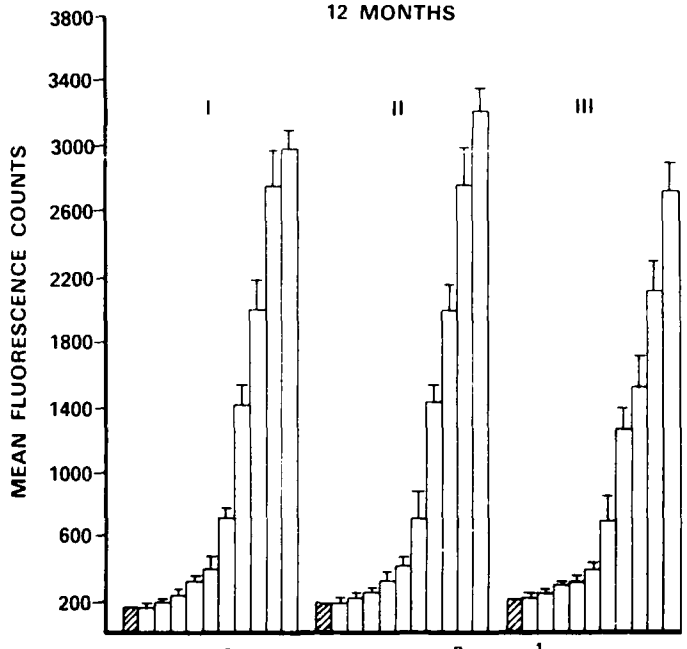

Figure 1e

Figure 1 (cont'd)

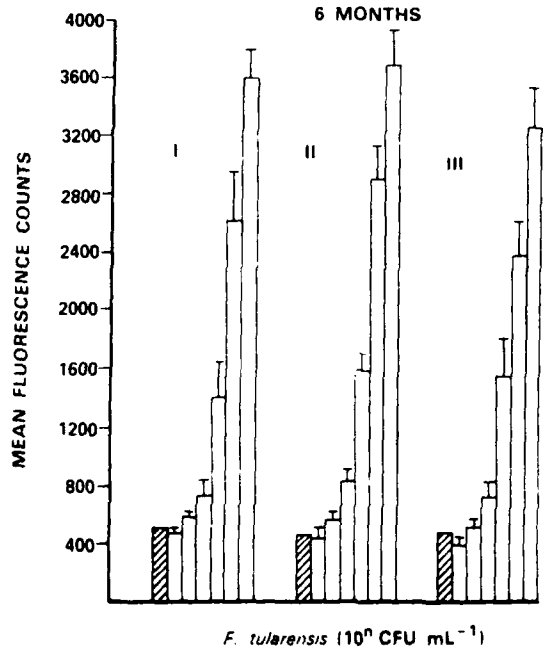

Figure 2b

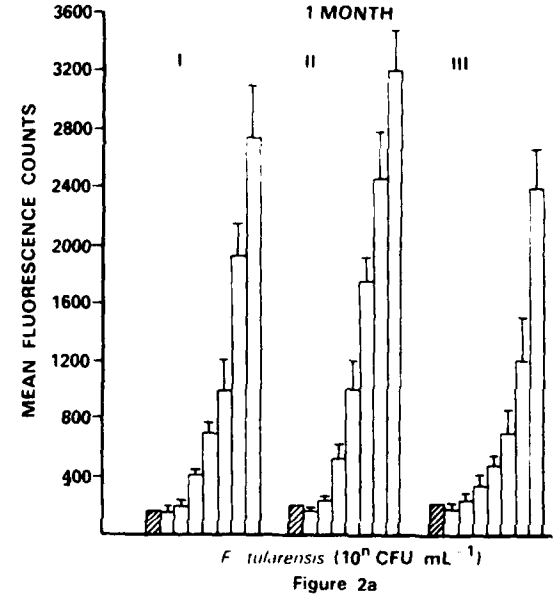

Figure 2a

Figure 2

Sensitivity of FELISA for the detection of *F. tularensis* whole cells on antibody-sensitized plates stored at −70° C for periods up to 1 yr. The system was challenged with log dilutions of *F. tularensis* whole cells (10^7 to 10^1 CFU mL^{-1}) in blocking buffer. Freshly prepared (control) plates (I), antibody-coated plates (II) and antibody-coated plates blocked with blocking reagent (III) were tested after 1, 6, 9 and 12 months (a-d, respectively). Data points are the mean of six determinations on a single plate. Error bars represent sd of the mean. (▨) negative control plus sd.

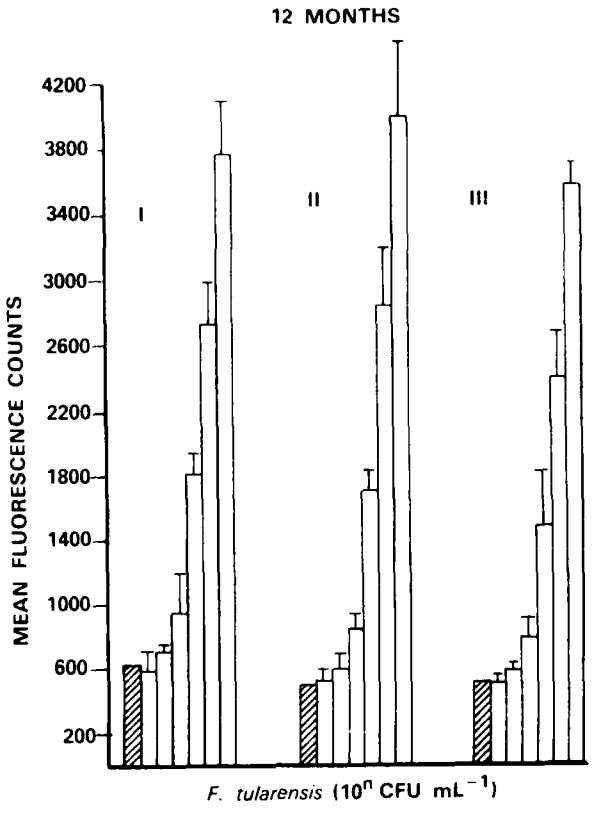

Figure 2d

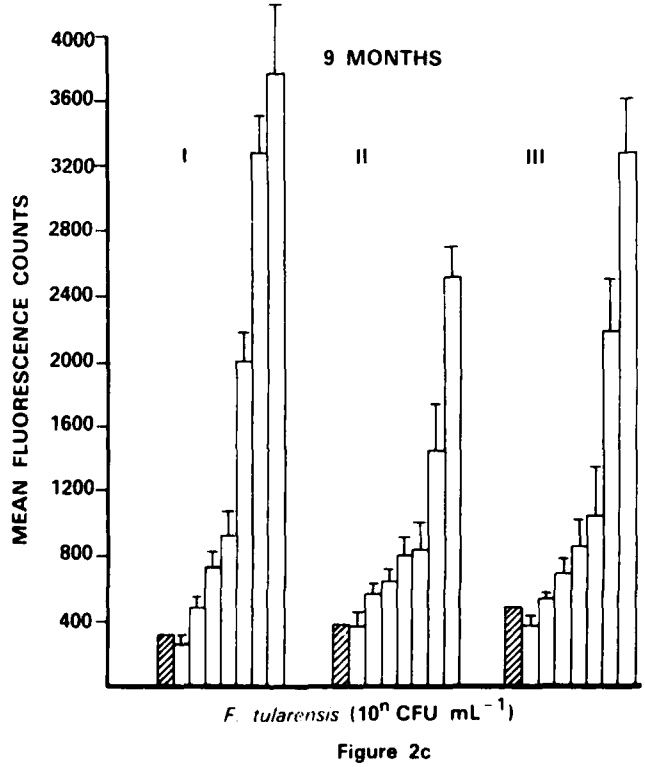

Figure 2c

Figure 2 (cont'd)

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This report describes a modified procedure for the "sandwich" fluorogenic enzyme-linked immunosorbent assay (FELISA) for the detection and quantitation of antigen, in which immunoassay plates were pre-coated with capture antibody or, alternatively, with capture antibody and blocking reagent, and stored frozen for periods up to 1 yr. Using Francisella tularensis as a model, two different identification protocols were employed. In the first, antibody-sensitized immunoassay plates stored frozen at -70°C were challenged with whole cells or with outer membrane protein of F. tularensis. In these experiments, there was no loss of sensitivity for the detection and quantitation of the antigen, when compared with freshly prepared immunoassay plates. This procedure took 5 h compared to more than 6 h required for performance of the assay using freshly prepared plates. In the second protocol, antibody-coated immunoassay plates were blocked with bovine serum albumin and stored frozen at -70°C for periods of up to 1 yr. This latter procedure shortened the assay time to 3 h while maintaining the same degree of sensitivity (10 fg (10^{-14} g) mL^{-1} of outer membrane protein and 100 colony forming units of F. tularensis mL^{-1}) as that achieved with freshly coated plates.

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Francisella tularensis

Outer membrane protein